Express Mail No. EV4MO5 53 65US

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 35/30, 35/14, C12P 21/00, C12N 5/06, 1/38

A1

(11) International Publication Number: WO 97/26901

(43) International Publication Date: 31 July 1997 (31.07.97)

(21) International Application Number: PCT/IB97/00051

(22) International Filing Date: 24 January 1997 (24.01.97)

(30) Priority Data:
116922 26 January 1996 (26.01.96) IL

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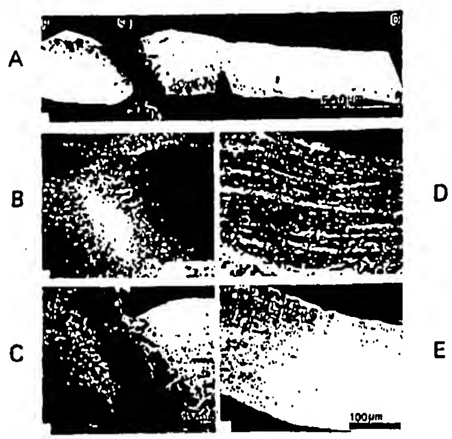
(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: REGENERATION OF NERVES IN THE CENTRAL NERVOUS SYSTEM



(57) Abstract

A central nervous system (CNS) injury is treated by transplanting into the site of the CNS injury a therapeutically effective amount of astrocytes which were pre-treated by exposing them *in vitro* to inflammation-associated cytokines.

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REGENERATION OF NERVES IN THE CENTRAL NERVOUS SYSTEM

FIELD OF THE INVENTION

The present invention is generally in the field of treatment of disorders and injuries in the central nervous system. More specifically, the present invention concerns a method and preparation for inducing or improving the regenerative process of central nervous system nerves.

PRIOR ART

The following is a list of prior art considered to be relevant as a background to the invention:

1. Sivron, T., Schwartz, M., *Glia*, 13:157-165 (1994).
2. Smith, G.M., Miller, R.H., *Brain Res.*, 543:111-22 (1991).
3. Wujek, J.R., Reier, P.J., *J. Comp. Neurol.*, 222:607-19 (1984).
4. Battisti, W.P., Wang, J., Bozek, K, Murray, M., *J. Comp. Neurol.*, 354:306-20 (1995).
5. Blaugrund, E., Lavie, V., Cohen, I., Solomon, A., Schreyer, D.J., Schwartz, M., *J. Comp. Neurol.*, 330:105-12 (1993).
6. Cohen, I., Shani, Y., Blaugrund, E., Schwartz, M., *Brain Res. Mol. Brain Res.*, 11:181-5 (1991).

7. Cohen, I., Shani, Y., Schwartz, M., *J. Comp. Neurol*, 334:431-43 (1993).
8. Lurie, D.I., Pijak, D.S., Selzer, M.E., *J. Comp. Neurol.*, 344:559-80 (1994).
9. Fishell, G., Hatten, M.E., *Development*, 113:755-65 (1991).
10. Smith, G.M., Miller, R.H., Silver, J., *J. Comp. Neurol.*, 251:23-43 (1986).
11. Smith, G.M., Rutishauser U., Silver, J., Miller R.H., *Dev. Biol.*, 138:377-90 (1990).
12. Smith, G.M., Silver, J., *Prog. Brain Res.*, (1988).
13. Faber-Elman, A.S.A., Abraham, J.A., and Schwartz, M., *J. Clin. Invest.*, 97:1-10 (1996).
14. Faber-Elman, V.L.A., Schvartz, I., Shaltiel, S, and Schwartz, M., *FASEB*, (1995).
15. Gadient, R.A., Cron, K.C., Otten, U., *Neurosci. Lett.*, 117:335-40 (1990).
16. Faber-Elman, A., Miskin, R., Schwartz, M., "*J. Neurochem.*, 65:1524-1535 (1995).
17. Araéjo, D.M., Cotman, C.W., *J. Neurosci.*, 12:1668-1678 (1992).
18. Eitan, S., Solomon, A., Lavie, V., Yoles, E., Hirschberg, D.L., Belkin, M., Schwartz, M., *Sciences*, 264:1764-1768 (1994).
19. Vigé, X., Tang, B., Wise, B.C., *Brain Res.*, 591:345-350.
20. Hirschberg, D.L., Schwartz, M., *J. Neuroimmunol.*, 61:89-96 (1995).
21. Perry, V.H., Crown, M.C., Gordon S., *J. Exp. Med.*, 165:1218-1223 (1987).

The references from the above list will be acknowledged in the text below by indicating their number in the list.

BACKGROUND OF THE INVENTION

Failure of the central nervous system (CNS) of adult mammals to regenerate after injury has been attributed, at least in part, to the failure of its molecular and cellular milieu to acquire growth-supportive properties. In the CNS of adult mammals, initial outgrowth from transected axons elongates for no more than a few millimeters. The failure of these nerves to regenerate is a reflection of the hostile and non-supportive character of the axonal environment created by cellular elements such as astrocytes, oligodendrocytes, blood-borne macrophages and resident microglia. Astrocytes are among the most prominent of the cellular elements, but their role in the process of regrowth is still unclear. The scar tissue formed by astrocytes in the injured mammalian CNS has been considered as a physical and/or biochemical barrier to regeneration[1-3], yet injured axons need astrocyte support for their growth and elongation across the site of the injury and beyond it[4-10]. Immature astrocytes, unlike mature ones, were shown to serve as a substrate for axonal growth *in vivo*[2,11,12]. In the peripheral nervous system (PNS), schwann cell processes extend from the cut end of axotomized nerves and are believed to guide the growing peripheral axons towards their target for reinnervation. It has been previously proposed that in the adult mammalian CNS both scar formation and the apparent failure of astrocytes to support regrowth may be linked to the failure of astrocytes to acquire essential post injury characteristics, such as migratory ability and expression of growth-supportive factors[13].

The ability of astrocytes to acquire such properties and hence allow regeneration depends on the nature of their immediate cellular and molecular environment[13-15]. Most of the environmental elements likely to be involved in modulating astrocyte behavior after injury are products of the inflammatory reaction. It was shown that *in vitro* incubation of astrocytes with cytokines affect a range of activities of these cells including production of proteases[16], production of growth factors and expression of extracellular molecules[15,17-19], and migratory ability[13,14]. Unlike in other tissues, however, the inflammatory response in the injured CNS is inadequate, at least temporarily[20,21].

SUMMARY OF THE INVENTION

In accordance with the invention it has been found that the inappropriate astrocyte response to injury in the CNS is a reflection of the inadequate inflammatory response and is not an expression of intrinsic properties of mature astrocytes. Thus, in accordance with the invention, astrocytes are incubated *in vitro* with a source for inflammation-associated cytokines and these pre-treated astrocytes are then transplanted into the site of injury in the CNS.

The present invention thus provides a method for treatment of a central nervous system (CNS) injury comprising exposing astrocytes *in vitro* to inflammation-associated cytokines and then transplanting a therapeutically effective amount of the astrocytes into a site of injury in the CNS.

The present invention provides in particular a method for inducing regeneration of injured nerves in the CNS comprising exposing astrocytes *in vitro* to inflammation-associated cytokines and then transplanting a therapeutically effective amount of the activated astrocytes into the CNS at a site comprising the injured nerves.

The exposure of the cytokines *in vitro* to inflammation-associated cytokines may be achieved in a number of ways. In accordance with one embodiment, this is achieved by incubating the astrocytes with a preparation comprising one or more inflammation-associated cytokines. Examples of cytokines are interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), a combination of IL-1β and TNF-α, a basic fibroblast growth factor (bFGF), and others. In accordance with another embodiment of the invention, the astrocytes are incubated with a preparation comprising cells capable of production and secretion of inflammation-associated cytokines, e.g. microglial cells or macrophages which have been activated by prior incubation with tissue, tissue fragments or cells capable of undergoing a process of regeneration, such as skin cells or tissue, peripheral nerves or

peripheral nerve tissue, liver cells or tissue, intestinal cells or tissue, etc. In addition, in accordance with a further embodiment of the invention, the astrocytes are incubated with a medium conditioned by cells which secrete inflammation-associated cytokines (e.g. the aforementioned activated microglial cells or macrophages), or with a fractionation product of such a conditioned medium (e.g. a concentrated proteinaceous fraction of such a conditioned medium, etc.). By a further embodiment of the invention, the astrocytes are incubated both with cells capable of production and secretion of inflammation-associated cytokines and with a non-cellular preparation comprising inflammation-associated cytokines (a pure cytokine preparation, the aforementioned conditioned medium or a fractionation product thereof, and a combination of these).

The transplantation of the astrocytes is preferably followed by irradiating the site of neural injury with a monochromatic light, e.g. from a laser source, such light is known to attenuate the post injury degenerative process. The light irradiation may at times be topical, i.e. shone onto skin portions overlaying the injury site. At times, where the site of injury is deep within the CNS and not accessible by topical light irradiation, the light may be channeled to the site of injury by implanted light guides.

Suitable light irradiation is at a wavelength of 632.8 nm. Typically, light irradiation will be for 3-4 minutes each day for 6-7 consecutive days. The light irradiation is typically a continuous light irradiation.

The present invention further provides a composition for use in the treatment of CNS injuries, in particular for inducing or accelerating regeneration of CNS nerves, comprising a therapeutically effective amount of astrocytes pre-treated by exposure to inflammation-associated cytokines, and a medium being physiologically compatible with the astrocytes and with the CNS environment.

The present invention still further provides use of astrocytes exposed *in vitro* with inflammation-associated cytokines for the preparation of a composition for transplantation or inoculation into an injured site of the

CNS, and particularly for inducing or accelerating regeneration of nerves in the CNS.

The present invention also provides a process for preparing a composition for the treatment of CNS injuries comprising treating astrocytes *in vitro* by exposing them to inflammation-associated cytokines and then mixing the astrocytes with a medium being physiologically compatible both with the astrocytes and with the CNS environment.

The invention still further provides a kit for the performance of the above process comprising one or more inflammation-associated cytokines, astrocytes compatible growth medium, and a physiologically compatible carrier medium for transferring the astrocytes to the injured site of the CNS. The kit may also comprise vessels for growing the astrocytes and for exposing them to the cytokines.

The astrocytes may be syngeneic astrocytes obtained from the individual, e.g. by means of a CNS biopsy. Preferably, and given the fact that the CNS is an immuno-privileged site, the astrocytes may also be allogeneic e.g. obtained from an astrocyte culture, from cadaver or even xenogeneic such as from a shark.

The exposure of the astrocytes to the inflammation-associated cytokines should be within an amount of such cytokines and for a time sufficient to activate the astrocytes so as to give rise to an anti-inflammatory response by the astrocytes. For example, in the case of IL-1β and TNF-α, a suitable amount may be about 800 U/ml and about 400 U/ml respectively, and the incubation with the cytokines may be for about 48-72 hrs.

After said exposure, the astrocytes may be formulated into a composition for transplantation into the CNS. The composition comprises an amount of astrocytes effective in achieving a desired therapeutic effect, e.g. nerve regeneration. The term *"therapeutic effective amount"* should be understood as meaning an amount of astrocytes required for achieving such an effect. What constitutes a therapeutic effective amount is easily attainable by the artisan without the need for undue experimentation, and depends on a number of factors including the site of injury within the CNS,

the type of injury, the severity of the injury, the time lapse from the injury until treatment, the age of the individual being treated, and others. The composition will further comprise a medium which is physiologically compatible with both the astrocytes as well as with the CNS. Such a medium may, for example, be saline, Dulbecco's Minimal Essential Medium (DMEM), L-15, etc.

The transplantation of the astrocytes into the CNS may typically be by inoculation. Other modes of transplanting may include causing the cells to attach to a solid substrate, e.g. membranes or beads and then transplanting the substrate with the attached cells into the CNS.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows staining of the nerve at the site of a nerve cut demonstrating that the nerve was detransected:

Fig. 1A is a microscopic view of the transected optic nerve. The left optic nerve of an adult rat was exposed under the dissecting microscope. A hole was made in the meninges and the nerve was transected. The procedure was photographed using a video camera. The transparent appearance of the lesion site can be seen;

Figs. 1B and **C** are micrographs showing retrograde labeling of retinal ganglion cells 24 hours after the optic nerve transection. In Fig. 1B the dye was applied 5 mm distal to the site of transection whereas in Fig. 1C the dye was applied directly to the site of transection.

Fig. 2 shows the distribution of transplanted astrocytes in the transected optic nerve of adult rat. Primary astrocyte cultures were prepared from 1-day postnatal rat cortex as described. The astrocytes were incubated with Hoechst stain (10.7 μM) for 10 min. at 37°C and washed twice with medium. Rat optic nerve was completely transected and immediately after the transection 2 ml of medium containing 2×10^5 astrocytes were transplanted into the site of injury. Seven days later optic nerves were excised and sliced:

Fig. 2A shows a longitudinal section through the transected nerve stained with GFAP antibodies at low magnification (P-proximal; D-distal; SI-site of injury);

Fig. 2B shows fluorescence of Hoechst-stained astrocytes at the site of transection;

Fig. 2C is a high magnification of the SI region of Fig. 2A;

Fig. 2D shows fluorescence in the optic nerve distal to the site of injury, evidencing migration of the astrocytes away from the site of injury; and

Fig. 2E shows staining of the same slice with GFAP antibodies.

Fig. 3 shows the recovery of VEP response in injured animals transplanted with treated astrocytes. Rat optic nerves were transected and transplanted either with astrocytes pre-treated with a combination of IL-1β and TNF-α or with untreated astrocytes. Electrodes were implanted in the visual cortex prior to the injury and visual evoked potential (VEP) responses were recorded from the left eye (injured) before injury and at 2, 7 and 9 weeks after injury. The recordings shown in the left column (under "A") are from an optic nerve transplanted with treated astrocytes and the recordings in the right column (under "B") are from an optic nerve transplanted with untreated astrocytes. Values are means of three recordings of 60 light flashes each. The areas around the lines denotes the standard error. The figure shows one example of each of these two groups. As can be seen there is a recovery in the treated nerve but not in the untreated nerve.

Fig. 4 shows exemplary pattern of VEP response recovery in eight animals:

Fig. 4A shows results recorded 9 weeks after transection and transplantation with pre-treated astrocytes except for rat no. 4, recorded after 13 weeks. The recording was performed with the contralateral (right) eye masked. In rat no. 8, the VEP response was recorded after cut of the right optic nerve. In all animals at three weeks after injury complete loss of VEP was recorded;

Fig. 4B shows average latencies of recovered nerves and uninjured nerves. Note the shift towards longer latency in the recovery nerves.

Fig. 5 shows a unitary action potential recorded from injured nerve transplanted with IL-1β and TNF-α treated astrocytes.

Fig. 6 shows retrograde labeling of retinal ganglion cells following VEP response recovery. A rat with transected optic nerves transplanted with the cytokine-activated astrocytes VEP recording was followed by application of lipophilic dye (dye 4-Di-10-Asp) applied 2 mm distal to the site of injury. Seven days later the retina was removed and cleaned of vitreous labeled ganglion cells were counted and photographed.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be illustrated with reference to experiments performed in adult rats in which the optic nerve has been completely transected. In these experiments, astrocytes pre-treated *in vitro* with inflammation-associated cytokines were transplanted into the site of injury and the effect of such transplantation on the recovery was assessed. The recovery was assessed by measurement of the visual evoked potential (VEP) response and the unitary action potential as well as in a morphological analysis by retrograde labeling.

EXPERIMENTAL METHODS

I. Electrodes:

The electrodes were gold contact pins (Wire-Pro, Inc., U.S.A.) soldered to screws, which were screwed into the holes and cemented to the skull with acrylic cement.

II. Light stimulation:

The stroboscopic light had the following characteristics: xenon flash tube 4 W/sec, 1-2 msec duration, 0.3 Hz), amplified 1000 times (AM Systems, microelectrode AC amplifier, model 1800) and digitized (12 bits,

5000 samples/sec) (National Instruments, Board NB-MIO16-9 and Lav View 2.2.1 data acquisition and analysis software).

III. Surgical procedure:

The left optic nerve was exposed through a small opening in the meninges. The nerve fibers were completely transected 3 mm from the globe, without damage to the nerve vasculature and with minimal damage to the meninges, by the use of a specially designed glass probe (dissector) with a 200 μm tip and a smooth blunt edge. To transect the nerve, the tip of the dissector was moved horizontally from right to left while being introduced slowly throughout the depth of the nerve until the whole width was traversed. The meninges surrounding the nerve were kept intact, except for the opening through which the dissecting probe was inserted. Medium (2 μl), with or without cells, was introduced via a glass pipet through the meningeal opening. This was followed by daily laser irradiation for 5 consecutive days, which was found to attenuate the post injury process of degeneration (Assia, E., *et al.*, *Brain Res.*, 476:205-212 (1988)). The laser was a helium-neon laser, irradiating light at 633 nm, the irradiation being for 3-4 mins. at an intensity of 7 mW.]

IV. Astrocyte-culture and pretreatment in the cytokines:

Astrocyte cultures were prepared as follows: Cells dissociated from the cerebral cortex of 1-day-old rats were cultured in poly-D-lysine-coated tissue culture flasks (two brains/85-cm^2 flask) containing Dulbecco's modified Eagle's medium, 2 mM glutamine 100 U/ml penicillin-0.1 mg/ml streptomycin, and 7.5% fetal calf serum. The medium was changed after 24 hours and every 2 days thereafter. To obtain pure cultures of astrocytes, after 8 days the flasks were shaken at 37°C on a rotary platform for 8 hours to remove macrophages, and afterwards for 16 hours to remove oligodendrocyte progenitors and type-2 astrocytes. Fresh medium was again added to the flasks. One day later, 25 mM cytosine-β-D-arabino-furanoside (Sigma, St. Louis, U.S.A.) was added. After 24 hours the medium was

replaced by defined medium, consisting of 2 mM glutamine, 0.1 mg/ml transferrin, 0.1% free fatty acid bovine serum albumin (BSA), 0.1 mM putrescine, 0.45 mM L-thyroxine, and 0.224 mM sodium selenite. The medium was replaced after 24 hours by fresh medium devoid of BSA, and either 800 U/ml IL-1β, 400 U/ml TNF-α or both those cytokines was added and incubated for 48 hrs.

V. Staining:

The lipophilic neurotracer dye 4-Di-10-Asp (4-(4-didecylamino)styrol-N-methylpyridinium iodide) (Molecular Probes, Europe BV) was applied to the injured optic nerve 2 mm from the distal border of the injury site (7-8 mm from the globe). The site of injury by this time was about 1-2 mm in diameter and was visible by its grayish color in comparison to the rest of the nerve, which maintained its original color. One week later the retina was removed, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined by fluorescence microscopy.

VI. Measurement of unitary potentials:

The VEP response at 8 weeks was followed by removal of the implanted electrodes from the visual cortex. The skull was carefully removed above the primary visual cortex. Unitary activity was measured using glass coated tungsten microelectrodes and the same amplifiers and data acquisition system described for the VEP measurements.

EXPERIMENTAL RESULTS

Electrodes were implanted in the visual cortex of anesthetized adult male rats (Sprague-Dawley (SPD), 8-9 weeks old) and baseline measurements of the VEP response were recorded. The left optic nerve was then completely transected.

Fig. 1A shows the transected nerve at the time of transection. The transection procedure leaves the meninges almost in tact; the axons are transected and the gap can be seen between the two cut ends of the nerve's

fiber bundles. Transection was also demonstrated by the lack of retrograde-labeled retinol ganglion cells with the neurotracer dye 4-Di-10-Asp was applied 24 hours after injury, 5 mm distal to the lesion site (Fig. 3B). Application of the dye to the site of transection resulted in heavy labeling (Fig. 3C). These results imply that the transection is complete or nearly complete.

Mature astrocytes from rat brain were pre-treated with cytokines, and immediately after transection were transplanted into the site of injury (2×10^5 cells/nerve in 2 μl medium). Transected nerves that were not transplanted or were transplanted with untreated mature astrocytes were used as controls. Fig. 2 shows a longitudinal section of transected nerve transplanted with astrocytes that were pre-treated with Hoechst stain and excised 1 week later. The nerve sections were also stained with anti GFAP antibodies to delineate the site of transection. The transplanted astrocytes are stained more faintly for GFAP and thus are distinguishable from nerve-resident astrocytes (Fig. 2B). The transplanted Hoechst stain-labeled astrocytes are shown in Fig. 2A. The cells remained viable at their transplantation site and some of them migrated along the *dura septa* from the site of application to the proximal and distal stumps of the nerve (Fig. 2C and D). The transplanted astrocytes remained alive for up to 6 weeks after injury, the latest time point examined (data not shown).

VEP responses were recorded at 2, 7 and 9 weeks after transection and treatment. At 2 weeks after transection, no positive VEP response could be detected in any of the animals. At 7 weeks, signs of recovery started to appear in nerves transplanted with astrocytes pre-treated with a combination of IL-1$\beta$and TNF-α. No positive response was detected in either of the control groups, nor in animals transplanted with astrocytes pre-treated with IL-1β or TNF-α alone. A VEP response in a transected optic nerve inoculated with pre-treated astrocytes as compared to such transfected with astrocytes which were not pre-treated is shown in Fig. 3. A total of 34 animals were transected and transplanted with astrocytes pre-treated with the combination of IL-1β and TNF-α. Seven weeks after injury and

treatment positive VEP responses were obtained in 11 animals, representing a range of 10 to 60% in five different experiments summarized in the following Table 1:

Table 1

Experiment No.	% positive VEP response
1	60
2	45
3	20
4	10
5	40

In all five experiments there was no positive VEP response in any of the controls (transplanted with non-treated astrocytes). In all animals that showed signs of recovery, the response became progressively stronger at 7 and 8 weeks. Some examples of positive VEP responses are shown in Fig. 4A. The response latencies in the recovered animals are longer than they were prior to transection (Fig. 4B). Fig. 5 presents a unitary action potential response at 8 weeks in the recovered nerve shown in Fig. 3A. Its long latency (about 80 msec) is in agreement with that observed for the recovered VEP response (about 100 msec). In nerves of the control groups, which showed no recovery of VEP response, no unitary action potential response could be detected.

Retinas from treated and control animals were also analyzed by retrograde labeling with the lipophilic neurotracer dye 4-Di-10-Asp, which was applied distally to the site of the injury 8-12 weeks after transection. One week later the retinas were excised, prepared as whole mounts, and analyzed. It can be reasonably assumed that only these fibers that had crossed the transection site as far as the site of dye application could take up the dye and retrogradely transport it to the ganglion cells. Labeled retinal ganglion cells were detected only in the retinas of nerves that had

been transplanted with astrocytes pre-treated with a combination of IL-1β- and TNF-α- (Fig. 6). In control animals, no retinal ganglion cells were labeled.

Qualitatively similar results to those reported above, although with less pronounced regeneration were also obtained after pre-exposure of the astrocytes to bFGF.

CLAIMS:

1. A method for treatment of a central nervous system (CNS) injury comprising exposing astrocytes *in vitro* to inflammation-associated cytokines and then transplanting a therapeutically effective amount of the astrocytes into a site of injury in the CNS.

2. A method for inducing regeneration of injured nerves in the CNS comprising exposing astrocytes *in vitro* to inflammation-associated cytokines and then transplanting a therapeutically effective amount of the astrocytes into the CNS at a site comprising the injured nerves.

3. A method according to Claim 1 or 2, comprising irradiating the site of injury after transplantation with a monochromatic light.

4. A method according to Claim 3, wherein said light is a laser light.

5. A method according to Claim 1 or 2, wherein said cytokines are a combination of interleukin-1β and tumor necrosis factor-α.

6. A method according to any one of Claims 1-5, comprising incubating the astrocytes *in vitro* with a preparation comprising inflammation-associated cytokines.

7. A method according to any one of Claims 1-5, comprising incubating the astrocytes *in vitro* with cells capable of secretion of inflammation-associated cytokines.

8. A composition for use in the treatment of central nervous system (CNS) injuries, comprising a therapeutically effective amount of astrocytes pre-treated by exposure to inflammation-associated cytokines and comprising a medium being physiologically compatible with the astrocytes and with the CNS environment.

9. A composition according to Claim 8, comprising astrocytes pre-treated with a combination of interleukin-1β and tumor necrosis factor-α.

10. Use of astrocytes exposed *in vitro* with inflammation-associated cytokines for the preparation of a composition for transplantation or inoculation into an injured site of the central nervous system.

11. A process for preparing a composition for the treatment of CNS injuries comprising treating astrocytes *in vitro* by exposing them to inflammation-associated cytokines and then mixing the astrocytes with a medium being physiologically compatible both with the astrocytes and with the CNS environment.

12. A process according to Claim 11, comprising exposing the astrocytes to a combination of interleukin-1β and tumor necrosis factor-α.

13. A kit for use in the process of Claim 11 or 12, comprising one or more inflammation-associated cytokines, astrocytes compatible growth medium and a physiological compatible carrier medium for transferring the astrocytes to the injured site within the CNS.

14. A method for inducing regeneration of an injured nerve in the CNS comprising transplanting a therapeutically effective amount of astrocytes into the CNS at a site of injured nerve, said astrocytes having been exposed to macrophages that have been stimulated by exposure to a peripheral nerve tissue or a tissue, tissue fragment or cells capable of undergoing spontaneous recovery following injury.

15. The method according to claim 14 further comprising transplanting an effective amount of macrophages with the astrocytes, said macrophages having been stimulated by exposure to a peripheral nerve tissue or a tissue, tissue fragment or cells capable of undergoing spontaneous recovery following injury.

1/8

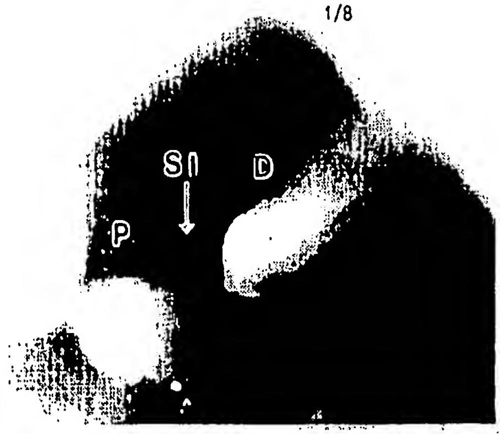


FIG.1A

FIG.1B

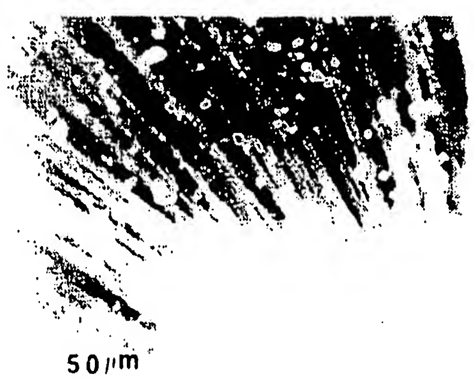


FIG.1C

2/8

FIG.2D FIG.2E

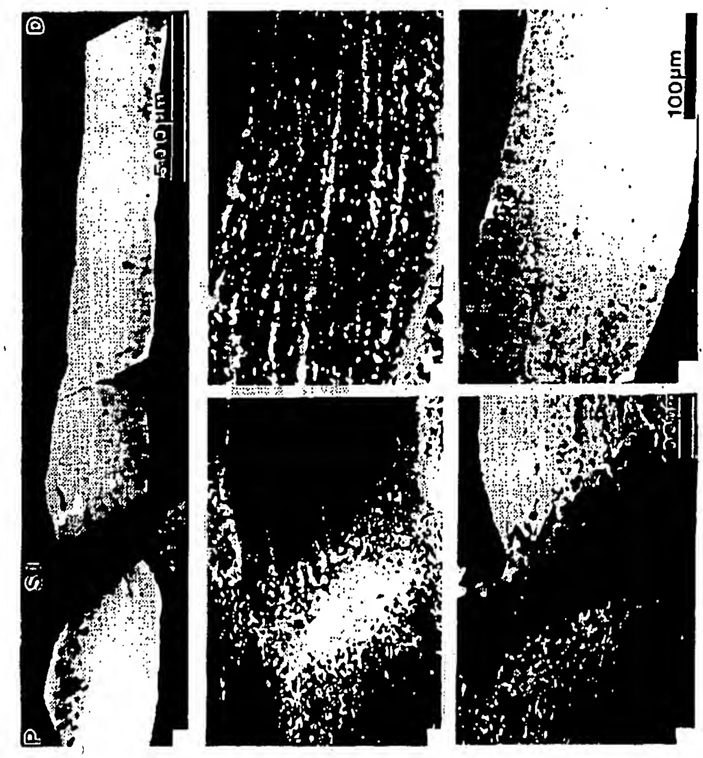
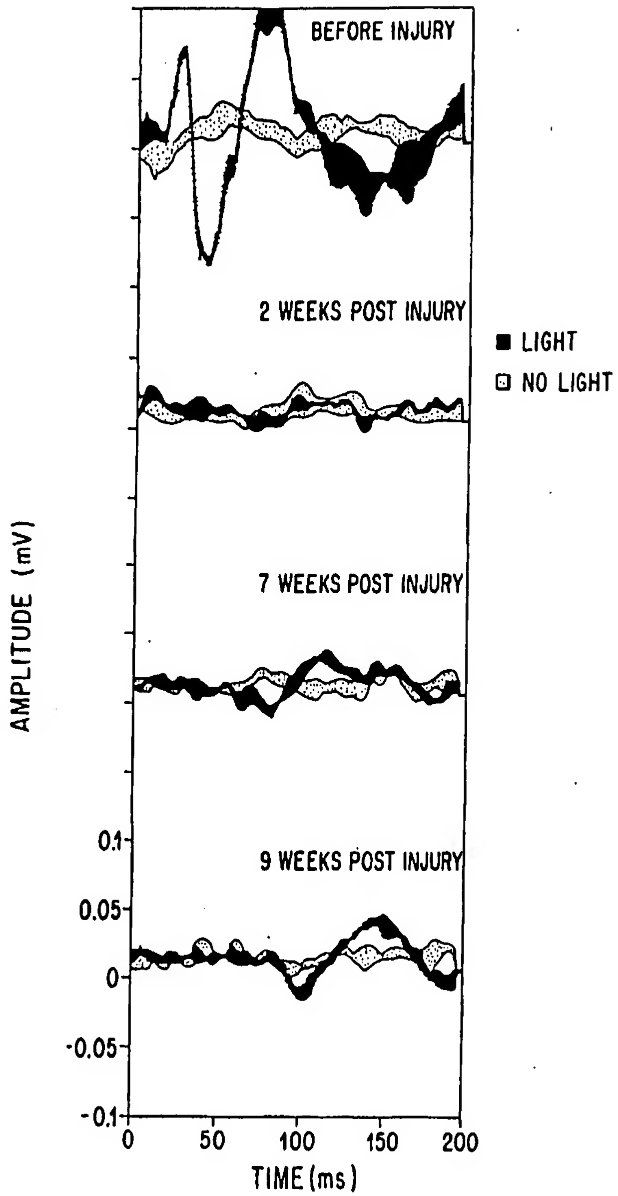


FIG.2A FIG.2B FIG.2C


BEFORE INJURY
2 WEEKS POST INJURY
7 WEEKS POST INJURY
9 WEEKS POST INJURY
LIGHT
NO LIGHT
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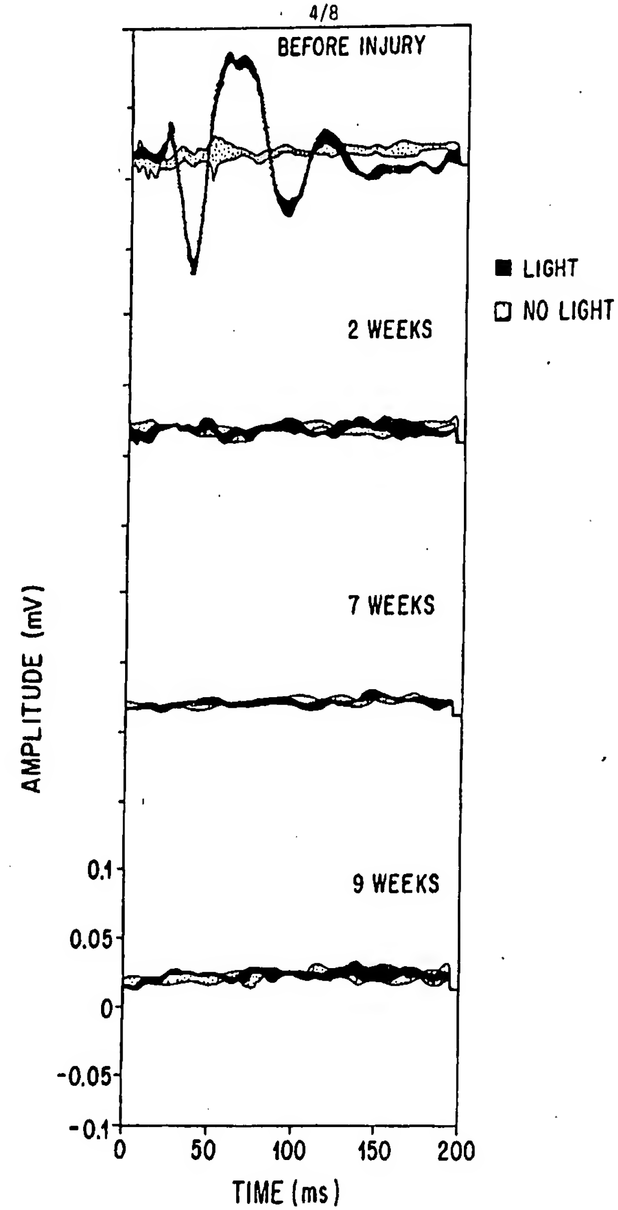
0.05
0
-0.05
-0.1
0
50
100
150
200
TIME (ms)

FIG. 3A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2 WEEKS
7 WEEKS
9 WEEKS
LIGHT
NO LIGHT
AMPLITUDE (mV)
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0.05
0
-0.05
-0.1
0
50
100
150
200
TIME (ms)

FIG. 3B

FIG. 4A

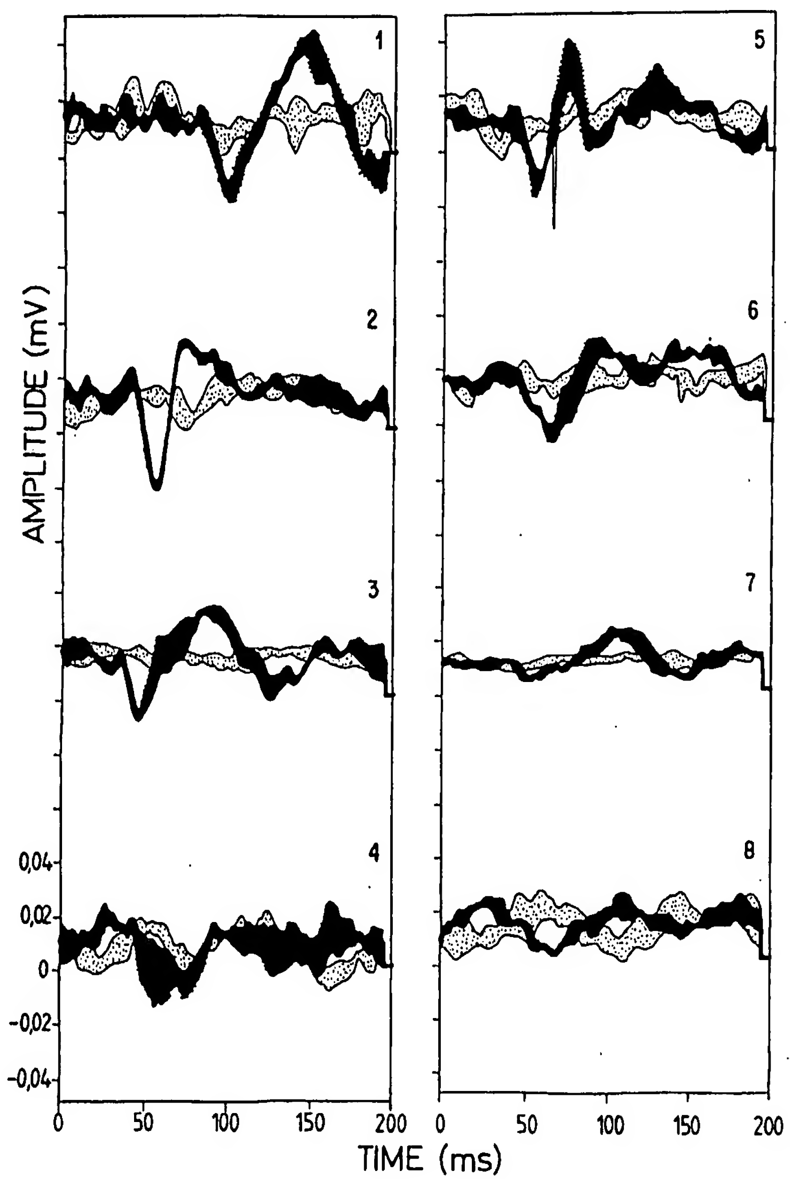
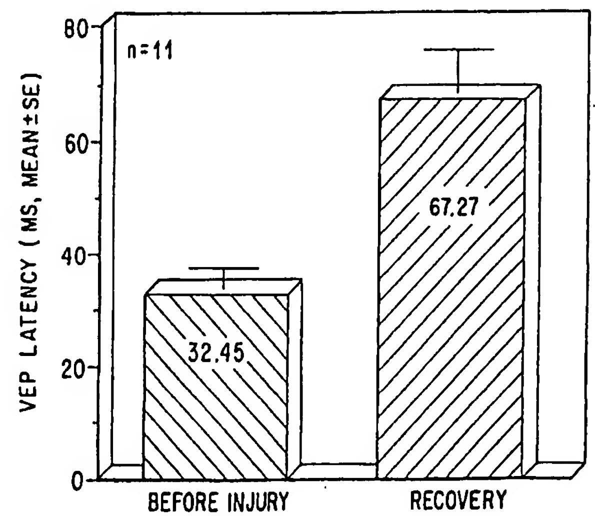

1
2
3
4
5
6
7
8
AMPLITUDE (mV)
0,04
0,02
0
-0,02
-0,04
0
50
100
150
200
TIME (ms)

FIG. 4B


n=11
VEP LATENCY (MS, MEAN±SE)
80
60
40
20
0
32.45
67.27
BEFORE INJURY
RECOVERY

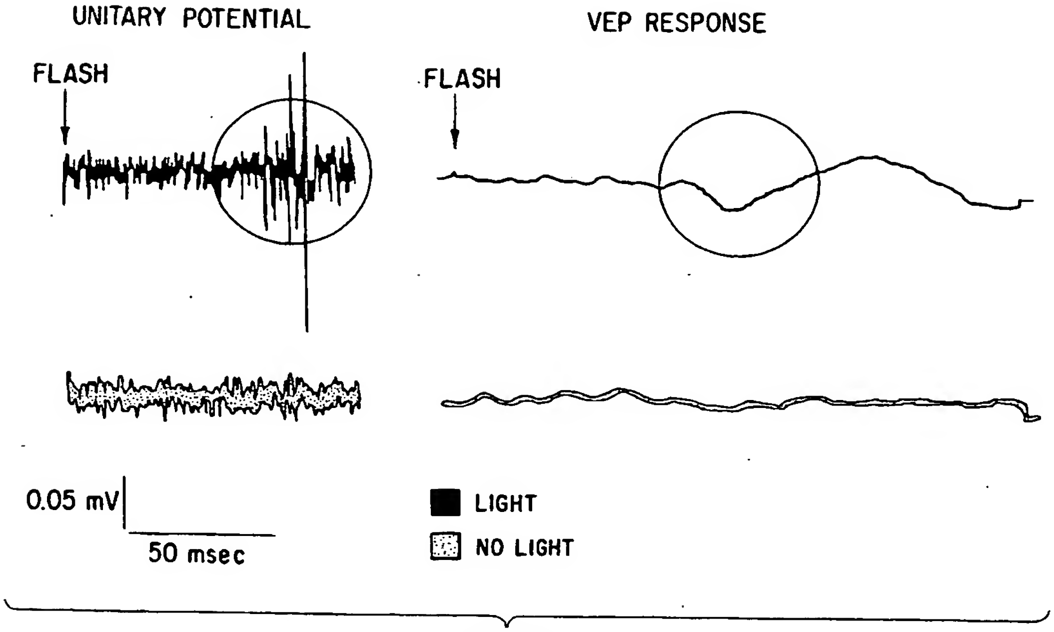

UNITARY POTENTIAL
VEP RESPONSE
FLASH
FLASH
0.05 mV
50 msec
LIGHT
NO LIGHT

FIG.5

FIG.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB97/00051

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	Faber-Elman, A. et al. Involvement of Wound-associated Factors in Rat Brain Astrocyte Migratory Response to Axonal Injury: In Vitro Simulation, J. Clin. Invest., 01 January 1996, Volume 97, Number 1, pages 162-171, see entire document.	8,10,11,13 — 1-5,9,12,14-15
Y	Schwartz, M. et al. Tumor Necrosis Factor Facilitates Regeneration of Injured Central Nervous System Axons, Brain Research, 05 April 1991, Volume 545, pages 334-338, see entire document.	1-5, 8-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 MAY 1997	09 JUN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jacqueline Krikorian Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB97/00051

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Lotan, M. et al. Cytokines Modulate the Inflammatory Response and Change Permissiveness to Neuronal Adhesion in Injured Mammalian Central Nervous System, Experimental Neurology, April 1994, Volume 126, pages 284-290, see entire document.	1-5, 8-15
Y	Blaugrund, E. et al. Disappearance of Astrocytes and Invasion of Macrophages Following Crush Injury of Adult Rodent Optic Nerves: Implications for Regeneration, Experimental Neurology, 08 October 1992, Volume 118, pages 105-115, see entire document.	1-5, 8-15
P, Y	Lazarov-Spiegler, O. et al. Transplantation of Activated Macrophages Overcomes Central Nervous System Regrowth Failure, FASEB J. September 1996, pages 1296-1302, see entire document.	14, 15
Y	Assia, E. et al. Temporal Parameters of Low Energy Laser Irradiation for Optimal Delay of Post-traumatic Degeneration of Rat Optic Nerve, Brain Research, 09 January 1989, Volume 476, pages 205-212, see entire document.	3, 4

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6 and 7
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB97/00051

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 35/30, 35/14; C12P 21/00; C12N 5/06, 1/38

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.1, 93.21, 93.3, 93.7; 435/70.1, 240.2, 244

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Medline, Medtext
Search terms: astrocyte?, glia?, nerv?, neuron?, regenerat?, grow?, regrow?, cytokine?, interleukin?, tnf?, macrophage?